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**First report of *Phyllorhiza punctata* von Lendenfeld, 1884 (Cnidaria: Scyphozoa, Mastigiidae) in the Balearic Islands (Western Mediterranean Sea)**

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## Materials and Methods

### Study area and data collection

The new findings of this study correspond to the coastal lagoon “Estany des Ponts” (Fig. 1) within the Albufera de Mallorca in the Balearic Islands. This area was a part of the wetlands of Alcudia Bay until its alteration in the nineteenth century. The area is included in the “*Inventario Balear de Zonas Húmedas*”. This lagoon is currently under pressure due to anthropogenic impact (WWF, 2018).

Since May 2023, monthly surveys have been conducted to search for *P. punctata* individuals in the area (either by spotting them from the shore or snorkelling) while measuring physicochemical variables (salinity and temperature). During these surveys, the specimens spotted were photographed for further processing with ImageJ v1.54 software to determine the bell size. Due to logistic issues, the genetic analysis was only carried out with only

one individual (Table 1). The specimen was collected by hand, trying to avoid damaging the body when removing it from the water (Fig. 2A). After collection, a piece of the organism was placed into 96% Ethanol ( $\text{CH}_3\text{CH}_2\text{OH}$ ) for molecular analysis; the rest of the individual was preserved in 4% Formaldehyde ( $\text{CH}_2\text{O}$ ) for morphological inspection. Samples were transported to the laboratory where they were stored at room temperature and  $-20\text{ }^{\circ}\text{C}$  for further processing.

### Molecular analyses

The DNA extraction was carried out on samples of tissue using the commercial Macherey-Nagel DNA Tissue extraction kit (Düren, Germany), following the manufacturer’s instructions. The quality and concentration of the DNA were measured using the Nanodrop ND1000 (Thermo Scientific, Waltham, MA, USA).



**Fig. 1:** Study area and finding location of *Phyllorhiza punctata*.

**Table 1.** New findings of *P. punctata* in the Balearic Islands.

Sighting date	Population status ( <i>P. punctata</i> )	Salinity range (PSU)	Temperature range ( $^{\circ}\text{C}$ )	Umbrella diameter (mm)	GeneBank Accession number
08-05-2023	-	26.1-28.6	23.4-25.6	-	-
08-06-2023	-	28.8-31.4	23.9-28.8	-	-
12-07-2023	15-20 specimens	32.7-36.5	28.5-33.0	20–25	-
12-08-2023	Single specimen	35.5-37.9	24.7-28.6	77	LC814843
13-09-2023	-	33.9-36.1	26.3-28.8	-	-
11-10-2023	-	32.6-35.6	23.0-26.1	-	-
17-01-2024	-	32.7-36.0	13.7-15.1	-	-
08-03-2024	-	27.5-28.8	15.4-17.0	-	-





**Fig. 2:** Data collection during surveys within the study area. A-Sample collection of *Phyllorhiza punctata* adult individual. B-Example of anthropogenic waste found during the surveys.

For species identification, a fragment of approximately 650 bp of the mitochondrial cytochrome c oxidase subunit I gene (barcoding) was amplified by PCR and sequenced using the universal primers HCO2198-LCO1490 (Folmer *et al.*, 1994). PCR reactions were performed in a total volume of 20  $\mu$ L containing 10  $\mu$ L KAPA Taq Ready Mix PCR kit (Sigma-Aldrich, Burlington, MA, USA), 0.4  $\mu$ L of each primer (stock 20  $\mu$ M), and 1  $\mu$ L of DNA (20 up to 80 ng/ $\mu$ L) and water to make up the final volume. The temperature protocol consisted of an initial denaturation step at 94  $^{\circ}$ C for 2 min, and 40 cycles of 94  $^{\circ}$ C for 30 s, 50  $^{\circ}$ C for 20 s, and 72  $^{\circ}$ C for 1 min. PCR products were separated on 1.5% agarose in TAE 1x buffer gels, including a HighRanger 1 kb DNA ladder size standard, stained with GelRed (Biotium, Fremont, CA, USA) (Norgen, Thorold, Canada), and visualized on a UV transilluminator.

Amplicons were extracted from the gels and purified using a mi-PCR purification Kit (Metabion International, Planegg, Germany) following the manufacturer's instructions. All PCR fragments were bi-directionally sequenced using the 3130xl DNA automated sequencer (Applied Biosystems, Carlsbad, CA, USA) at Secugen S.L. service (Madrid, Spain). Finally, the sequences were edited and aligned using BioEdit v7.2.5 software (Hall, 1999) to perform a comparative analysis with other known sequences in GenBank, using the BLAST application.

## Results and Discussion

The consensus sequences were deposited in GenBank under accession number LC814843. BLAST results showed similarity with other sequences obtained from the Balearic waters, particularly with sequences of *Phyllorhiza* spp. (99.2% identity; Access. numb. MT904380) and *Phyllorhiza punctata* (99.05% identity; Access. numb. GQ120101).

In the approximately 600 bp fragment analyzed, we detected 10 nucleotide differences compared to the *P. punctata* sequence present in GenBank. Furthermore, we observed double peaks at eight nucleotide positions, specifically indicated as Y (C or T) at positions 144, 303, and 447, or R (A or G) at positions 273, 333, 336, 447, and 483 of the previously reported sequence. These findings suggest the presence of potential heteroplasmy within

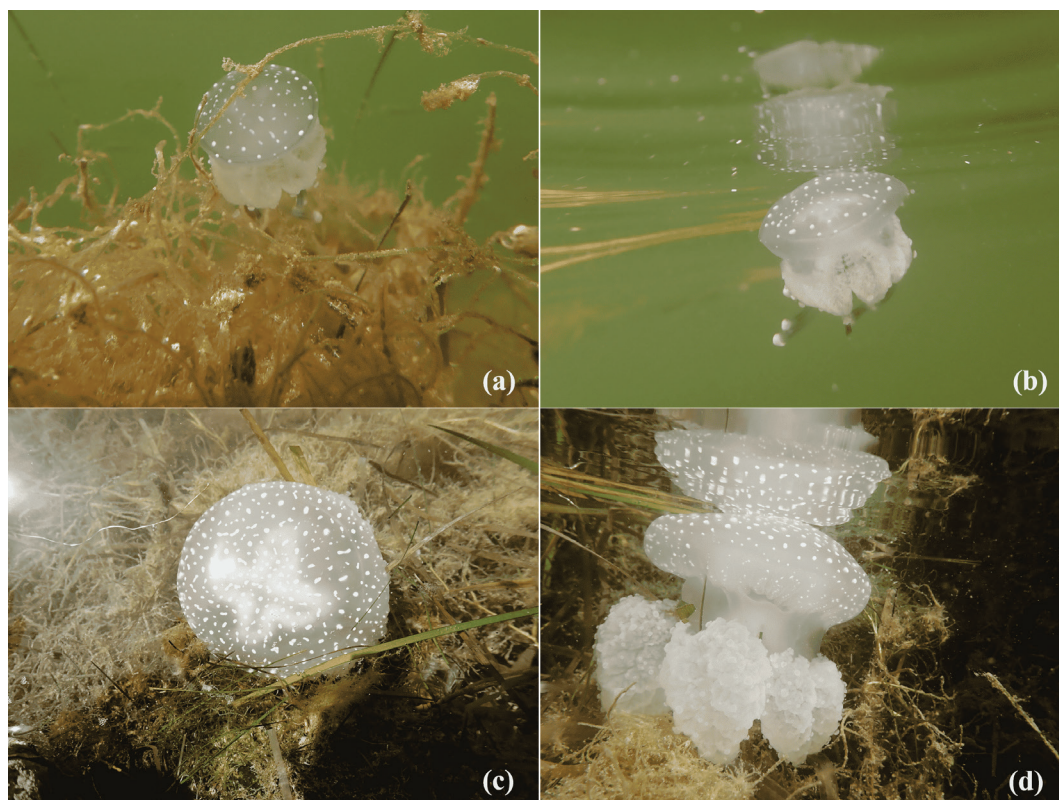
the species as seen in other invertebrate species (Rodríguez-Pena *et al.*, 2020). However, more samples should be analyzed to confirm this hypothesis.

Since May 2023, eight surveys have been carried out in the study area; *P. punctata* individuals were spotted only in two of these surveys (Table 1). The range of bell sizes varied from 20 to 77 mm. No specimens over 77 mm have been found in this location. This is in contrast to reports from other regions, where umbrella diameters exceed 100 mm (Graham *et al.*, 2003; Abed-Navandi & Kikinger, 2007; Galil *et al.*, 2009; Enrique-Navarro & Prieto, 2020).

The bell size of the young individuals spotted in July (20-25mm) was similar to that of juvenile individuals found in Rio San Pedro tidal creek (RSP) (Cadiz, Spain); while the size of the single individual spotted in August was within the size of the adults found in RSP (Enrique-Navarro & Prieto, 2020). The small size and the low number of individuals observed may indicate a recent invasion of this area of the Mediterranean Sea and the absence of an established population, unlike other regions in the Mediterranean Sea where a high number of sexually mature specimens have been reported (Galil *et al.*, 2009; Gueroun *et al.*, 2015; Kaminas *et al.*, 2022).

The juvenile specimens photographed presented a blue pigmentation of the bell (Fig. 3A-B) and terminal clubs, similar to the juvenile individuals found at RSP (Enrique-Navarro & Prieto, 2020). Neither this pigmentation nor the light brown color background of the bell was found in the adult individual (Fig. 3C-D), although these are characteristic of the zooxanthellae symbiosis found in other adult individuals (Ocaña-Luna *et al.*, 2010; Gueroun *et al.*, 2015; Stamouli *et al.*, 2018). This morphological variability has been previously reported concerning the presence or absence of zooxanthellae (Bolton & Graham, 2004; Madkour *et al.*, 2021), whose absence leads *P. punctata* to feed on zooplankton (Graham *et al.*, 2003).

In the surveys conducted in the area, *P. punctata* individuals were only found in the summer months (Table 1), which may suggest a preference for the physicochemical properties of the water present during these months. A similar phenomenon has been reported in Mexico, where this species shows a seasonal pattern: their numbers rise in the spring and summer months and decline in the rainy and cold seasons, especially when the salinity is below



**Fig. 3:** *Phyllorhiza punctata* individuals found during the surveys in summer 2023. A-B, young individuals found in July 2023; C-D adult individual found in August 2023 on which the genetic analysis was performed.

10 psu (Ocaña-Luna *et al.*, 2010). Although this species tolerates a wide range of temperature and salinity conditions, the absence of individuals during the rainy and cold seasons in the Estany des Ponts may signal non-optimal conditions for the proliferation of *P. punctata* in surface waters, like in the Swan-Canning estuary in western Australia (Rippingale & Kelly, 1995). Unlike other regions, the shallow depth of Estany des Ponts limits *P. punctata* from seeking deeper shelter from alluvial rain and storms during the rainy and cold seasons (Martínez *et al.*, 1989). Nonetheless, *P. punctata* adults and ephyra individuals have been detected in shallow coastal lagoons such as the Mar Menor in Murcia (Fernández-Alías *et al.*, 2024). Thus, further monitoring is required to evaluate the evolution of *P. punctata*'s population in our study area in subsequent years, as the increase in temperature and salinity (especially in the summer months) may cause a sudden proliferation of this species.

Since its first detection in 1965 in Israel (Galil *et al.*, 1990), *P. punctata* seems to have spread and established in several regions of the eastern Mediterranean Sea (Galil *et al.*, 2009). In the western Mediterranean Sea, *P. punctata* was spotted for the first time in Italy in 2009 (Boero *et al.*, 2009). Three years later, it was detected for the first time on the coasts of Tunisia and Spain with more sightings in subsequent years (Barrado *et al.*, 2014; Gueroun *et al.*, 2015). While Madkour *et al.* (2021) anticipated the possible spread of this species into the western Mediterranean Sea, some possible mechanisms for *P. punctata*'s spread in the Mediterranean Sea have been proposed (Fernández-Alías *et al.*, 2024). This tropical jellyfish is among the top three most invasive species of the 45 jel-

lyfish species assessed by Killi *et al.*, (2020). Some of the reported impacts of *P. punctata* include decreased abundance of zooplankton and the clogging of nets in the shrimp industry (García & López, 1989; García, 1990; Ocaña-Luna *et al.*, 2010; Gueroun *et al.*, 2015).

*P. punctata*'s tolerance to changes in environmental parameters and its abundance in coastal zones such as estuaries, lagoons, and bays (Graham *et al.*, 2003) makes the monitoring of these environments necessary for the evaluation and management of this tropical species. As Fernández-Alías *et al.*, (2024) pointed out, the role of semi-enclosed habitats as stepping-stones in the *P. punctata* invasion of the Mediterranean Sea is crucial to understanding their colonization patterns (Carlton, 1996).

Our findings constitute the first record of this non-native species in the Balearic Islands and confirm the spread of the species throughout the Mediterranean Sea. Therefore, the monitoring of this species as well as studies on its population dynamics are necessary to establish and evaluate effective management and conservation strategies for the upcoming years.

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**Author contributions:** Conception, design, and collection of field data were conducted by RG and MVL. Analysis and interpretation of genetic data were performed by GC, while RG and MVL handled the environmental data. All authors contributed to the drafting of the initial manuscript, its subsequent revision, and the review and approval of the final version. **Funding:** This work was supported by the European Union's LIFE program through the project LIFE PINNARCA (NAT/ES/001265). **Data availability:** The data that support the findings of this study are available from the corresponding author upon reasonable request. **Conflicts of interest:** The authors declare no conflicts of interest.

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